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(71) Applicant: THE SALK INSTITUTE FOR BIOLOGICAL STUDIES (US/US); 10010 North Torrey Pines Road, La Jolla, CA 92037 (US).			
(72) Inventors: EVANS, Ronald, M.; 8615 La Jolla Village Drive North, La Jolla, CA 92037 (US). FOREMAN, Barry, M.; Apartment #299, 8568 Via La Jolla, La Jolla, CA 92037 (US). KLIEWER, Steven, A.; 104 Southwick Court, Cory, NC 27513 (US). ONG, Estelita, S.; 6307 Hannon Court, San Diego, CA 92117 (US).			
(74) Agent: REITER, Stephen, E.; Pretty, Schroeder, Brueggemann & Clark, Suite 2000, 444 South Flower Street, Los Angeles, CA 90071 (US).			Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: MAMMALIAN PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS AND USES THEREOF			
(57) Abstract			
<p>Peroxisome proliferator-activated receptor subunits designated PPARγ and PPARδ are described. Nucleic acid sequences encoding the receptor subunits, expression vectors containing such sequences and host cells transformed with such vectors are also disclosed, as are heterodimeric PPAR receptors comprising at least one of the invention subunits, and methods for the expression of such novel receptors, and various uses therefor.</p>			

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MAMMALIAN PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS AND USES THEREOF

RELATED APPLICATIONS

This application is a continuation-in-part of
5 United States Application Serial Number 08/270,643, now
pending, which is a continuation-in-part of United States
Application Serial Number 07/907,908, filed July 2, 1992,
now abandoned, which is a continuation-in-part of United
States Application Serial Number 07/497,935, filed March
10 22, 1990, now abandoned.

FIELD OF INVENTION

The present invention relates to novel members of
the steroid/thyroid superfamily of receptors, as well as
uses therefor.

15 BACKGROUND OF THE INVENTION

Peroxisome proliferators are a structurally
diverse group of compounds which, when administered to
rodents, elicit dramatic increases in the size and number
of hepatic and renal peroxisomes, as well as concomitant
20 increases in the capacity of peroxisomes to metabolize
fatty acids via increased expression of the enzymes
required for the β -oxidation cycle (Lazarow and Fujiki,
Ann. Rev. Cell Biol. 1:489-530 (1985); Vamecq and Draye,
Essays Biochem. 24:1115-225 (1989); and Nelali et al.,
25 *Cancer Res.* 48:5316-5324 (1988)). Chemicals included in
this group are the fibrate class of hypolipidemic drugs,
herbicides, and phthalate plasticizers (Reddy and Lalwani,
Crit. Rev. Toxicol. 12:1-58 (1983)). Peroxisome
proliferation can also be elicited by dietary or
30 physiological factors such as a high-fat diet and cold
acclimatization.

Insight into the mechanism whereby peroxisome proliferators exert their pleiotropic effects was provided by the identification of a member of the nuclear hormone receptor superfamily activated by these chemicals (Isseman and Green, *Nature* 347:645-650 (1990)). This receptor, termed peroxisome proliferator activated receptor alpha (PPAR α), was subsequently shown to be activated by a variety of medium and long-chain fatty acids and to stimulate expression of the genes encoding rat acyl-CoA oxidase and hydratase-dehydrogenase (enzymes required for peroxisomal β -oxidation), as well as rabbit cytochrome P450 4A6, a fatty acid *ω*-hydroxylase (Gottlicher et al., *Proc. Natl. Acad. Sci. USA* 89:4653-4657 (1992); Tugwood et al., *EMBO J.* 11:433-439 (1992); Bardot et al., *Biochem. Biophys. Res. Comm.* 192:37-45 (1993); Muerhoff et al., *J. Biol. Chem.* 267:19051-19053 (1992); and Marcus et al., *Proc. Natl. Acad. Sci. USA* 90(12):5723-5727 (1993). The foregoing references support a physiological role for PPAR α in the regulation of lipid metabolism. PPAR α activates transcription by binding to DNA sequence elements, termed peroxisome proliferator response elements (PPRE), as a heterodimer with the retinoid X receptor. The retinoid X receptor is activated by 9-*cis* retinoic acid (see Kliwer et al., *Nature* 358:771-774 (1992), Gearing et al., *Proc. Natl. Acad. Sci. USA* 90:1440-1444 (1993), Keller et al., *Proc. Natl. Acad. Sci. USA* 90:2160-2164 (1993), Heyman et al., *Cell* 68:397-406 (1992), and Levin et al., *Nature* 355:359-361 (1992)). Since the PPAR α -RXR complex can be activated by peroxisome proliferators and/or 9-*cis* retinoic acid, the retinoid and fatty acid signaling pathways are seen to converge in modulating lipid metabolism.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided isolated mammalian peroxisome proliferator-

activated receptor subunit proteins of the γ and δ subtypes, and functional fragments thereof. In addition, there are provided isolated nucleic acids encoding mammalian peroxisome proliferator-activated receptor subunit proteins, as well as fragments thereof. There are also provided vectors containing the above-described nucleic acids, as well as cells containing such nucleic acids and/or vectors.

The present invention also provides methods for the recombinant production of mammalian peroxisome proliferator-activated receptor proteins comprising at least one PPAR subunit protein of the γ and δ subtype, and functional fragments thereof, as well as methods to identify clones encoding the above-described receptor subunit proteins, and functional fragments thereof.

Also provided by the present invention are methods for screening compounds to determine those which bind to mammalian peroxisome proliferator-activated receptor proteins comprising at least one PPAR subunit protein of the γ or δ subtype, or functional fragments thereof, as well as bioassays for evaluating whether test compounds are agonists or antagonists for receptor proteins of the invention, or functional modified forms of said receptor protein(s).

25 BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents a schematic comparison of the members of the PPAR gene family using mPPAR δ as a reference. Comparisons among the different domains of the proteins are expressed as percent amino acid identity.

30 Figure 2 demonstrates that PPAR γ and PPAR δ fail to respond to the peroxisome proliferator Wy 14,643. CV-1 cells were cotransfected with reporter plasmid PPRE $_3$ -TK-LUC

and either no receptor expression plasmid (-), CMX-PPAR α , CMX-PPAR γ , or CMX-PPAR δ and then incubated in either the absence (-) or presence (+) of 5 μ M Wy 14,643. Luciferase activities are expressed as percentages of the maximal response where 100% is the activity obtained with PPAR α in the presence of 5 μ M Wy 14,643.

Figure 3 illustrates the ability of PPAR γ and PPAR δ to repress PPAR α -mediated responsiveness to Wy 14,643. CV-1 cells were cotransfected with reporter plasmid PPRE₃-TK-LUC and either no receptor expression plasmid (NONE) or CMX-PPAR α (10ng) in either the absence or presence of CMX-PPAR γ (100ng) or CMX-PPAR δ (100ng). Cells were then incubated in either the absence (-) or presence (+) of 5 μ M Wy 14,643. Luciferase activities are presented as fold-activation relative to cells which were not transfected with receptor expression plasmid and were not treated with Wy 14,643.

Figure 4 demonstrates that PPAR isoforms are pharmacologically distinct. CV-1 cells were cotransfected with reporter plasmid PPRE₃-TK-LUC and either no receptor expression plasmid (-), CMX-PPAR α , CMX-PPAR γ , or CMX-PPAR δ in either the absence or presence of 5 μ M Wy 14,643 (WY), 30 μ M linoleic acid (C18:2), or 30 μ M LY-171883 (LY). Luciferase activities are presented as the fold activation achieved in compound-treated versus mock-treated cells. Similar results were obtained in triplicate in three independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

Two novel PPAR receptor subunits have been cloned and characterized. These novel γ and δ isoforms (subunits) together with the α subunit display marked differences in their responsiveness to peroxisome proliferators and fatty acids, as well as differences in their temporal and spatial

patterns of expression. These observations suggest a broad role for the PPAR family during development and in adult physiology.

The existence of multiple PPAR isoforms with
5 distinct expression patterns has been found to correlate with the fact that the three isoforms have different ligand specificities. Indeed, the PPAR isoforms are shown herein to be pharmacologically distinct. Thus, PPAR α , PPAR γ and PPAR δ are most efficiently activated by Wy 14,643,
10 LY-171883, and linoleic acid, respectively. Remarkably, Wy 14,643, which results in approximately 100-fold induction in reporter expression in the presence of PPAR α , fails to activate either PPAR γ or PPAR δ .

With regard to this differential responsiveness
15 to activators of peroxisome proliferation, the relationship among the PPAR isoforms may be analogous to that between the glucocorticoid and mineralocorticoid receptors (GR and MR, respectively). While both receptors can bind to the same response element, and both respond to
20 mineralocorticoids and corticosteroids, MR and GR display differential sensitivities to aldosterone and specific glucocorticoids such as dexamethasone, respectively (Arriza et al., *Neuron* 1:887-900 (1988)). Thus, the ratio of these receptors to their ligands provides a means of determining
25 tissue-specific expression of target genes. Similarly, the existence of multiple PPAR isoforms with overlapping ligand specificities may provide the means for tissue-specific regulation of gene expression by peroxisome proliferators and fatty acids.

30 In addition to their differential responsiveness to peroxisome proliferators, the three PPAR isoforms also display distinct yet overlapping expression patterns. As previously shown, PPAR α mRNA is abundant in liver and kidney (Isseman and Green, supra; Beck et al., *Proc. R.*

- Soc. Lond.* 247:83-87 (1992)), tissues in which peroxisome proliferators result in dramatic increases in the numbers of peroxisomes and concomitant increases in peroxisomal β -oxidation (Nemali et al., supra). In contrast, the
- 5 levels of PPAR γ mRNA and PPAR δ mRNA, which can act as dominant repressors of PPAR α -mediated responsiveness to Wy 14,643, are low in these tissues. Thus, a pattern emerges in which tissues that are most responsive to peroxisome proliferators such as Wy 14,643 are observed to
- 10 express high amounts of PPAR α mRNA and relatively low amounts of PPAR γ mRNA and PPAR δ mRNA. These data suggest that the ratio of the PPAR isoforms is likely to play a critical role in establishing the degree of responsiveness of tissues to specific peroxisome proliferators.
- 15 Widespread expression of PPAR δ is observed in both the embryo and in adult tissues. This observation suggests that this isoform may play a general "housekeeping" role. In contrast, PPAR γ is observed to be expressed almost exclusively in the adrenal and spleen.
- 20 The expression of all three PPAR isoforms in the adrenal is particularly intriguing, since diseases which result in peroxisome dysfunction (e.g. adrenoleukodystrophy and Zellweger syndrome) cause gross morphological changes in adrenal cells and, eventually, adrenal deficiency. These
- 25 observations suggest a critical role for peroxisomes in this tissue (Vamecq and Draye, supra). Interestingly, peroxisomes can be induced to proliferate in hamster adrenals in response to treatment with adrenocorticotrophic hormone and corticosteroids (Black and Russo, *Amer. J.*
- 30 *Anatomy* 159:85-120 (1980)), indicating the presence of adrenal-specific signaling pathway(s) for peroxisome proliferation. The differential expression of PPAR γ in the adrenal suggests that this isoform may respond to an adrenal-enriched ligand.

Accordingly, in accordance with the present invention, there are provided isolated mammalian peroxisome proliferator-activated receptor subunit proteins of the γ or δ subtype and functional fragments thereof.

5 As employed herein, the phrase "mammalian peroxisome proliferator-activated receptor subunit proteins of the γ or δ subtype" refers to isolated and substantially purified as well as recombinantly produced proteins which are members of the steroid/thyroid superfamily of
10 receptors, and which mediate the pleiotropic effects of peroxisome proliferators (such as medium and long-chain fatty acids). Such receptors participate in the formation of heterodimeric species with retinoid X receptors (RXRs) and comprise an amino-terminal domain, a DNA binding
15 domain, and a ligand binding domain. Also contemplated within this definition are variants thereof encoded by mRNA generated by alternative splicing of a primary transcript.

 Use of the terms "recombinantly produced", "isolated" or "substantially pure" in the present
20 specification and claims as a modifier of DNA, RNA, polypeptides or proteins means that the modified substances have been produced by the hand of man, and thus are separated from their native in vivo cellular environment. As a result of this human intervention, the recombinant/
25 isolated/substantially pure DNAs, RNAs, polypeptides and proteins of the invention are useful in ways that the naturally occurring DNAs, RNAs, polypeptides or proteins are not, for example, in assays to identify selective drugs or compounds.

30 The novel receptors of the present invention also can be included as part of a panel of receptors which are screened to determine the selectivity of interaction of proposed agonists or antagonists of other steroid hormone

receptors. Thus, a compound which is believed to interact selectively, for example, with the glucocorticoid receptor, should not have any substantial effect on any other receptors, including invention receptors. However, if such
5 a proposed compound does interact with the invention receptors, then the probability of side effects caused by the activation of other receptors in addition to the target receptor, is clearly indicated. For example, the use of many drugs in the treatment of hormone-related disorders is
10 currently restricted by side effects caused by the activation of "non-target" receptors. Employment of the invention receptors in a panel of receptors in a screen to determine the selectivity of interaction of potential ligands provides a means to identify receptor-specific
15 ligands that are therapeutically superior than currently used ligands that cause unwanted side effects.

As used herein, the term splice variant refers to variant PPAR encoding nucleic acid(s) produced by differential processing of primary transcript(s) of genomic
20 DNA, resulting in the production of more than one mRNA. cDNA derived from differentially processed primary transcript will encode PPAR receptor proteins that have regions of complete amino acid identity and regions having different amino acid sequences. Thus, the same genomic
25 sequence can lead to the production of multiple, related mRNAs and corresponding proteins. Both the resulting mRNAs and proteins are referred to herein as "splice variants".

Accordingly, also contemplated within the scope of the present invention are nucleic acids that encode
30 mammalian PPAR receptor subunit proteins as defined above, but that by virtue a degenerate genetic code do not necessarily hybridize to the nucleic acids set forth in SEQ ID NOS: 1 or 3 under specific hybridization conditions. Nucleic acid fragments encoding invention receptor subunit
35 proteins are capable of forming a functional heterodimer

with one or more RXR receptor protein isoform(s). Typically, unless a PPAR receptor protein is encoded by mRNA that arises from alternative splicing (i.e., a splice variant), PPAR receptor encoding DNA and encoded protein
5 share substantial sequence homology with at least one of the PPAR receptor-encoding DNAs and encoded proteins described herein. It is understood that DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but
10 include regions of nearly 100% homology to a DNA fragment described herein, and encode an open reading frame that includes start and stop codons and encodes a functional PPAR receptor protein.

Exemplary nucleic acid sequences encoding
15 mammalian peroxisome proliferator-activated receptor subunit proteins of the γ subtype are represented by nucleotide sequences which encode substantially the same amino acid sequence as set forth in SEQ ID NO:2. Presently preferred sequences encode the same amino acid sequence as
20 set forth in SEQ ID NO:2.

Exemplary nucleic acid sequences can alternatively be characterized as those nucleotide sequences which encode mammalian peroxisome proliferator-activated receptor subunit proteins of the γ subtype and
25 hybridize under high stringency conditions to SEQ ID NO:1.

Exemplary nucleic acid sequences encoding mammalian peroxisome proliferator-activated receptor subunit proteins of the δ subtype are represented by nucleotides which encode substantially the same amino acid
30 sequence as set forth in SEQ ID NO:4. Presently preferred sequences encode the same amino acid sequence as set forth in SEQ ID NO:4.

Especially preferred sequences are those which have substantially the same nucleotide sequence as that set forth in SEQ ID NO:1.

Exemplary nucleic acid sequences can alternatively be characterized as those nucleotide sequences which encode mammalian peroxisome proliferator-activated receptor subunit proteins of the δ subtype and hybridize under high stringency conditions to SEQ ID NO:3.

Especially preferred nucleic acid sequences are those which have substantially the same nucleotide sequence as the coding sequences in SEQ ID NO:3.

The phrase "stringency of hybridization" is used herein to refer to conditions under which polynucleic acid hybrids are stable. As known to those of skill in the art, the stability is reflected in the melting temperature (T_m) of the hybrids. T_m can be approximated by the formula:

$$81.5^{\circ}\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{G+C}) - 600/l,$$

where l is the length of the hybrid in number of nucleotides. T_m decreases approximately 1-1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is initially performed under conditions of low stringency, followed by washes of varying, but higher, stringency. Reference to hybridization stringency relates to such washing conditions. Thus, as used herein:

- (1) HIGH STRINGENCY refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C (i.e., if a hybrid is

- not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.1X SSPE, and 0.1% SDS at 65°C;
- (2) MODERATE STRINGENCY refers to conditions that permit hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 65°C; and
- (3) LOW STRINGENCY refers to conditions that permit hybridization in 10% formamide, 5X Denhart's solution, 6X SSPE, 0.2% SDS at 42°C, followed by washing in 1X SSPE, 0.2% SDS, at 50°C.

It is understood that these conditions may be varied using a variety of buffers and temperatures well known to skilled artisans.

As used herein, the phrase "substantial sequence homology" refers to nucleotide sequences which share at least about 90% identity, and amino acid sequences which typically share more than 95% amino acid identity. It is recognized, however, that proteins (and DNA or mRNA encoding such proteins) containing less than the above-described level of homology arising as splice variants or that are modified by conservative amino acid substitutions (or substitution of degenerate codons) are contemplated to be within the scope of the present invention.

As used herein, the phrase "substantially the same" refers to nucleotide sequences, ribonucleotide sequences, or amino acid sequences, that have slight and

non-consequential sequence variations from the actual sequences disclosed herein. Species that are "substantially the same" are considered to be equivalent to the disclosed sequences, and as such are within the scope of the appended claims. In this regard, "slight and non-consequential sequence variations" mean that sequences that are substantially the same as invention sequences disclosed and claimed herein, are functionally equivalent to the sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same results as the nucleic acid and amino acid sequences disclosed and claimed herein. Specifically, functionally equivalent nucleic acids encode proteins that have conservative amino acid variations, such as substitution of a non-polar residue for another non-polar residue or a charged residue for a similarly charged residue. These changes are recognized by those of skill in the art as modifications that do not substantially alter the tertiary structure of the protein.

Fragments of invention nucleic acid sequences are useful as hybridization probes, wherein such fragments comprise at least 14 contiguous nucleotides of the above-described nucleic acids, and wherein the fragment is labeled with a detectable substituent. Suitable detectable substituents can be readily determined by those of skill in the art, and include such species as radiolabeled molecules, fluorescent molecules, enzymes, ligands, and the like.

As used herein, a probe is single- or double-stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases that are the same as (or the complement of) any 14 or more contiguous bases set forth in SEQ ID NOs:1 or 3. Preferred regions for the construction of probes include those regions predicted to

encode a DNA binding domain. Such regions are preferred because they are most highly conserved among members of the steroid/thyroid superfamily of receptors.

As a particular application of the invention sequences, genetic screening can be carried out using the nucleic acid sequences of the invention as probes. Thus, nucleic acid samples from patients having conditions suspected of involving alteration/modification of any one or more of the PPAR receptor subtypes can be screened with appropriate probes to determine if abnormalities exist with respect to the endogenous PPAR receptor proteins.

In accordance with yet another embodiment of the present invention, there are provided vectors comprising nucleic acid sequences, as well as cells and vectors containing such sequences. Such host cells, including bacterial, yeast and mammalian cells can be used for expressing invention nucleic acids to produce PPAR receptor protein(s). Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes, and selection of transfected cells are well known in the art (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press). Heterologous DNA may be introduced into host cells by any method known to those of skill in the art, such as transfection by CaPO_4 precipitation with a vector encoding the heterologous DNA (see, e.g., Wigler et al. (1979) *Proc. Natl. Acad. Sci.* 76:1373-1376), DEAE-dextran, electroporation, microinjection, or lipofectamine (GIBCO BRL #18324-012). Transfected host cells can then be cultured under conditions whereby the receptor subunit protein(s) encoded by the DNA is (are) recombinantly expressed.

The present invention further provides a mammalian peroxisome proliferator-activated receptor, expressed recombinantly in a host cell. The receptor comprises at least one PPAR subunit, wherein the PPAR
5 subunit is PPAR γ or PPAR δ , and at least one retinoid X receptor isoform. The invention receptor has the ability to repress PPAR α -mediated responses activated by Wy 14,643,

Also provided by the present invention are mammalian peroxisome proliferator-activated subunit
10 proteins, expressed recombinantly in a host cell wherein the receptor subunits have substantially the same amino acid sequence as set forth in SEQ ID NOs: 2 or 4.

In accordance with still another embodiment of the present invention, there is provided a method for the
15 recombinant production of mammalian peroxisome proliferator-activated receptor proteins comprising at least one PPAR subunit of the γ or δ subtype, or functional fragments thereof. Such method comprises expressing the above-described nucleic acid(s) in a suitable host cell.

In accordance with still another embodiment of the present invention, there is provided a method to identify clones encoding mammalian peroxisome proliferator-activated receptor subunit proteins of the γ or δ subtype, or functional fragments thereof. Such method comprises
25 screening a genomic or cDNA library with an invention nucleic acid probe under low stringency hybridization conditions, and identifying those clones which display a substantial degree of hybridization to said fragment.

Nucleic acids encoding mammalian peroxisome
30 proliferator-activated receptor subunit protein of the γ or δ subtype, or functional fragments thereof may be isolated by screening suitable human cDNA or human genomic libraries under suitable hybridization conditions with nucleic acids

disclosed herein (including nucleotide sequences derived from SEQ ID NOS:1 or 3). Suitable libraries can be prepared from appropriate tissue samples, e.g., brain tissue, heart tissue, intestinal tissue, kidney tissue, 5 liver tissue, spleen tissue, and the like. The library can be screened with nucleic acid including substantially the entire receptor-encoding sequence thereof, or the library may be screened with a suitable probe, as described above.

After screening the library, positive clones are 10 identified by means of a hybridization signal; the identified clones are characterized by restriction enzyme mapping and/or DNA sequence analysis, and then examined, by comparison with the sequences set forth herein to ascertain whether they encode a complete PPAR receptor subunit 15 protein (i.e., if they include translation initiation and termination codons). If the selected clones are incomplete, they may be used to rescreen the same or a different library to obtain overlapping clones. If the library is genomic, then the overlapping clones may include 20 exons and introns. If the library is a cDNA library, then the overlapping clones will include an open reading frame. In both instances, complete clones may be identified by comparison with the DNA and encoded proteins provided herein.

25 The ligand-binding domain (LBD) of nuclear hormone receptors is a complex multifunctional unit containing subdomains for dimerization, transcriptional suppression and hormone-induced transactivation (Forman and Samuels, *Mol. Endocrinol.* 4:1293-1301 (1990)). The 30 dimerization domain includes a series of heptad repeats flanked by sequences required for ligand binding. Thus, the dimerization domain is embedded within the larger LBD. This structural arrangement raises the possibility that dimerization may serve as an allosteric modulator of ligand 35 binding and transactivation. It has previously been shown

that the *Drosophila* ecdysone receptor (EcR) acquires ligand binding activity after heterodimerization with USP (*Drosophila* homolog of RXR; see Yao et al., in *Nature* 366:476-479 (1993)). Thus, differential interactions among 5 receptor LBDs can either restrict, redirect or lead to an acquisition of new ligand binding phenotypes.

It has recently been shown that PPAR α binds to its cognate response elements as a heterodimer with the RXR (see Kliewer et al., supra, Gearing et al., supra, or 10 Keller et al., supra). The resulting PPAR α -RXR complex can respond to both peroxisome proliferators and 9-*cis* retinoic acid (see Kliewer et al., (1992), supra). It has now been found that PPAR γ and PPAR δ also cooperate with RXR in the formation of heterodimers, and in binding to DNA as 15 heterodimers. Ultimately, the regulation of peroxisome physiology is likely a consequence of a complex interplay among the multiple PPAR and RXR isoforms and the ligands for these receptors.

In accordance with the present invention, there 20 are provided combinations of receptors comprising at least two different members of the steroid/thyroid superfamily of receptors, wherein one receptor is either PPAR γ or PPAR δ , and wherein said receptors are associated in the form of a multimer, preferably a heterodimer. A particularly 25 preferred combination of receptors is a heterodimer comprising either PPAR γ or PPAR δ and a subtype of RXR.

Combinations contemplated by the present invention can broadly be referred to as "multimeric species," which is intended to embrace all of the various 30 oligomeric forms in which members of the steroid/thyroid superfamily of receptors (including fragments thereof comprising the dimerization domains thereof) are capable of associating in combination with either PPAR γ or PPAR δ . Thus, reference to "combinations" of steroid receptors or

"multimeric" forms of steroid receptors includes homodimeric combinations of a single PPAR γ or PPAR δ receptor (including fragments thereof comprising the dimerization domains thereof), heterodimeric combinations of either a PPAR γ or PPAR δ receptor and another different receptor (including fragments thereof comprising the dimerization domains thereof), homotrimeric combinations of a single PPAR γ or PPAR δ receptor (including fragments thereof comprising the dimerization domains thereof), heterotrimeric combinations of two or three different receptors including PPAR γ or PPAR δ (including fragments thereof comprising the dimerization domains thereof), homotetrameric combinations of a single PPAR γ or PPAR δ receptor (including fragments thereof comprising the dimerization domains thereof), heterotetrameric combinations of two or more different receptors including PPAR γ or PPAR δ (including fragments thereof comprising the dimerization domains thereof), and the like.

As employed herein, the phrase "members of the steroid/thyroid superfamily of receptors" (also known as "nuclear receptors" or "intracellular receptors") refers to hormone binding proteins that operate as ligand-dependent transcription factors, including identified members of the steroid/thyroid superfamily of receptors for which specific ligands have not yet been identified (referred to hereinafter as "orphan receptors"). These hormone binding proteins have the intrinsic ability to bind to specific DNA sequences. Following binding, the transcriptional activity of target gene (i.e., a gene associated with the specific DNA sequence) is modulated as a function of the ligand bound to the receptor.

The DNA-binding domains of all of these nuclear receptors are related, consisting of 66-68 amino acid residues, and possessing about 20 invariant amino acid residues, including nine cysteines. A member of the

superfamily can be identified as a protein which contains the above-mentioned invariant amino acid residues, which are part of the DNA-binding domain of such known steroid receptors as the human glucocorticoid receptor (amino acids 421-486), the estrogen receptor (amino acids 185-250), the mineralocorticoid receptor (amino acids 603-668), the human retinoic acid receptor (amino acids 88-153). The highly conserved amino acids of the DNA-binding domain of members of the superfamily are well-known as set forth, for example in PCT WO 94/01558. Thus, the DNA-binding domain is a minimum of 66 amino acids in length, but can contain several additional residues.

Exemplary members of the steroid/thyroid superfamily of receptors contemplated for use in the practice of the present invention (including the various isoforms thereof) include steroid receptors such as mineralocorticoid receptor, progesterone receptor, androgen receptor, vitamin D₃ receptor, and the like; plus retinoid receptors, such as the various isoforms of RAR (e.g., RAR α , RAR β , or RAR γ), the various isoforms of RXR (e.g., RXR α , RXR β , or RXR γ), and the like; thyroid receptors, such as TR α , TR β , and the like; as well as other gene products which, by their structure and properties, are considered to be members of the superfamily, as defined hereinabove, including the various isoforms thereof. Examples of orphan receptors include HNF4 [see, for example, Sladek et al., in *Genes & Development* 4: 2353-2365 (1990)], the COUP family of receptors [see, for example, Miyajima et al., in *Nucleic Acids Research* 16: 11057-11074 (1988), and Wang et al., in *Nature* 340: 163-166 (1989)], COUP-like receptors and COUP homologs, such as those described by Mlodzik et al., in *Cell* 60: 211-224 (1990) and Ladias et al., in *Science* 251: 561-565 (1991), the ultraspiracle receptor [see, for example, Oro et al., in *Nature* 347: 298-301 (1990)], and the like. Presently preferred members of the superfamily

for use in the practice of the present invention are the various isoforms of RXR (e.g., RXR α , RXR β , or RXR γ).

The formation of multimeric (e.g., heterodimeric) species can modulate the ability of the first receptor to trans-activate transcription of genes maintained under expression control in the presence of ligand for said first receptor. The actual effect on activation of transcription (i.e., enhancement or repression of transcription activity) will vary depending on the receptor species which is combined with either a PPAR γ or PPAR δ receptor to form the multimeric species, as well as on the response element with which the multimeric species interacts.

In accordance with the present invention, there are provided multimeric receptor species which belong to the steroid/thyroid superfamily of receptors, comprising at least the dimerization domain of at two different members of the steroid/thyroid superfamily of receptors, wherein one of the members is selected from the invention PPAR γ or PPAR δ .

As employed herein, the term "dimerization domain" of a member of the steroid/thyroid superfamily of receptors refers to that portion of the receptor which is believed to be involved in the formation of multimeric receptor species. This domain typically comprises the carboxy-terminal portion of the receptor, i.e., that portion of a receptor which is 3' with respect to the DNA-binding domain of the receptor. See, e.g., Evans, in *Science* 240:889-895 (1988), and Forman and Samuels, *Mol. Endocrinol.* 4:1293-1301 (1990). Presently preferred members of the superfamily for use in deriving the dimerization domain are the various isoforms of RXR (e.g., RXR α , RXR β , or RXR γ).

In accordance with the present invention, there are also provided heterodimer complexes comprising either PPAR γ or PPAR δ and a silent partner therefor.

As employed herein, the term "silent partner" refers to members of the steroid/thyroid superfamily of receptors which are capable of forming heterodimeric species with either PPAR γ or PPAR δ , wherein the silent partner of the heterodimer does not have any ligand bound to the ligand-binding domain (LBD) when the silent partner is complexed with a PPAR subtype (i.e., only the PPAR co-partner of the heterodimer binds ligand). Presently preferred silent partners for use in the practice of the present invention are the various isoforms of RXR (e.g., RXR α , RXR β , or RXR γ).

In accordance with a further embodiment of the present invention, there is provided a method for screening compounds to determine those which bind to mammalian peroxisome proliferator-activated receptor proteins comprising at least one PPAR subunit of the γ or δ subtype, or functional fragments thereof. Such method comprises employing receptor protein(s) of the invention in a binding assay, which comprises, contacting receptor protein(s) of the invention with test compound, and identifying those compounds which bind to invention receptor protein(s).

In accordance with a still further embodiment of the present invention, there is provided a bioassay for evaluating whether test compounds are agonists for receptor proteins of the invention, or functional modified forms of said receptor protein(s). Such bioassay comprises:

- (1) contacting suitable host cells expressing said receptor protein with test compound under physiological conditions, wherein said host cells contain DNA encoding a reporter

protein, wherein said DNA is operatively linked to a PPAR-response element;

- (2) monitoring said host cells for expression of reporter gene, wherein expression of reporter protein reflects transcriptional activity of the receptor protein and, therefore, the presence of an activated receptor-ligand complex.

In accordance with yet another embodiment of the present invention, there is provided a bioassay for evaluating whether test compounds are antagonists for receptor proteins of the invention, or functional modified forms of said receptor protein(s). Such bioassay comprises:

- contacting suitable host cells with
- (i) increasing concentrations of at least one compound whose ability to inhibit the transcription activation activity of agonists of mammalian peroxisome proliferator-activated receptor proteins of the γ or δ subtype is sought to be determined, and
- (ii) a fixed concentration of at least one agonist for said receptor protein(s) or functional modified forms thereof,
- wherein suitable test cells express mammalian peroxisome proliferator-activated receptor proteins comprising at least one PPAR subunit of the γ or δ subtype and DNA encoding a reporter protein, wherein said DNA is operatively linked to a PPAR-response element; and thereafter
- assaying for evidence of transcription of said reporter gene in said cells as a function of the concentration of said compound in said culture medium, thereby indicating the ability of

5 said compound to inhibit activation of transcription by agonists of mammalian peroxisome proliferator-activated receptor proteins comprising at least one PPAR subunit of the γ or δ subtype.

 In accordance with a still further embodiment of the present invention, there is provided a method for identifying ligands selective for heterodimers comprising either PPAR γ or PPAR δ and a silent partner therefor. Such
10 method comprises

 comparing the level of expression of reporter when cells containing a reporter construct, either PPAR γ or PPAR δ and silent partner therefor are exposed to test compound, relative to the level of expression of reporter
15 when cells containing a reporter construct, either PPAR γ or PPAR δ and a member of the steroid/thyroid superfamily which is not a silent partner therefor are exposed to test compound, and

 selecting those compounds which activate only the
20 combination of either PPAR γ or PPAR δ and silent partner therefor.

 In accordance with yet another embodiment of the present invention, there are provided antibodies generated against the invention proteins. Such antibodies can be
25 employed for studying receptor tissue localization, subunit composition, structure of functional domains, as well as in diagnostic applications, therapeutic applications, and the like. Preferably, for therapeutic applications, the antibodies employed will be monoclonal antibodies.

30 The above-described antibodies can be prepared employing standard techniques, as are well known to those of skill in the art, using the invention receptor proteins or portions thereof as antigens for antibody production. Both anti-peptide and anti-fusion protein antibodies can be

used [see, for example, Bahouth et al. (1991) Trends Pharmacol Sci. vol. 12:338-343; Current Protocols in Molecular Biology (Ausubel et al., eds.) John Wiley and Sons, New York (1989)]. Factors to consider in selecting
5 portions of the invention receptor protein subunit sequences for use as immunogen (as, for example, a synthetic peptide or a recombinantly produced bacterial fusion protein) include antigenicity, accessibility (i.e., internal or external domains), uniqueness to the particular
10 protein subunit, and the like.

The availability of sequence-specific antibodies enables use of immunohistochemical techniques to monitor the distribution and expression density of various protein subunits (e.g., in normal versus diseased brain tissue).
15 Such antibodies can also be employed for diagnostic and therapeutic applications.

In accordance with yet another embodiment of the present invention, there are provided methods for modulating processes mediated by mammalian peroxisome
20 proliferator-activated receptor proteins comprising at least one PPAR subunit of the γ or δ subtype. Such methods comprise contacting mammalian peroxisome proliferator-activated receptor proteins of the γ or δ subtype with an effective, modulating amount of agonist, antagonist or
25 antibody according to the present invention.

The antibodies, agonists and/or antagonists of the invention can be administered to a subject employing standard methods, such as, for example, by intraperitoneal, intramuscular, intravenous, or subcutaneous injection,
30 implant or transdermal modes of administration, and the like. One of skill in the art can readily determine dose forms, treatment regimens, etc, depending on the mode of administration employed.

Processes which are mediated by mammalian peroxisome proliferator-activated receptor proteins of the γ or δ subtype include, for example, macrophage production in the spleen which is believed to be important in atherosclerosis.

The invention will now be described in greater detail with reference to the following non-limiting examples.

EXAMPLE 1

Screening of cDNA libraries

PPAR γ was isolated by screening an adult mouse liver AZAP cDNA library (Stratagene) with a synthetic oligonucleotide (GGNTTYCAYTAYGGNGTNCAYCG; SEQ ID NO:5) under conditions previously described by Blumberg et al., in *Proc. Natl. Acad. Sci. USA* **89**:2321-2325 (1992). This oligonucleotide is a mixture of all possible DNA sequences encoding the amino acid sequence GFHYGVHA (SEQ ID NO:6), a sequence present in the loop of the first zinc finger in the *Xenopus* PPAR α , PPAR β and PPAR γ isoforms.

PPAR δ was isolated by screening an E6.5 mouse AZAPII cDNA library (a gift of D.E. Weng and J.D. Gerhart, Johns Hopkins University) under low stringency conditions with a cDNA fragment encoding the human retinoic acid receptor α cDNA binding domain (Mangelsdorf et al., *Nature* **345**:224-229 (1990)). In both screens, positive clones were converted to plasmids by the automatic excision process.

EXAMPLE 2

Cotransfection Assay

The mammalian expression vectors pCMX-PPAR α , pCMX-PPAR γ and pCMX-PPAR δ were constructed by inserting the cDNA inserts of PPAR α , PPAR γ , and PPAR δ into pCMX as

previously described by Umesono et al., in Cell 65:1255-1266 (1991)). Construction of the reporter PPRE₃-TK-LUC has also been previously described by Kliewer et al., (1992) supra. Cotransfection assays in CV-1 cells were done in 48 well plates using N-[1-(2,3-dioleoyloxy)-propyl]N,N,N-trimethyl ammonium methyl sulfate (DOTAP) according to the manufacturer's instructions (Boehringer Mannheim). Transfections contained 10ng of receptor expression plasmid vector, 20ng of the reporter PPRE₃-TK-LUC, 60ng of pCMX- β GAL (β -galactosidase) as an internal control, and 210ng of carrier plasmid pGEM. Cells were incubated in the presence of DOTAP for 8 hours, washed, and incubated in the presence of peroxisome proliferators or fatty acids for 36 hours. Cell extracts were prepared and assayed for luciferase and β -galactosidase activity as previously described (Umesono, supra). All experimental points were done in triplicate.

EXAMPLE 3

Northern Analysis

Preparation of poly(A)⁺ RNA from rat tissues and Northern analysis were performed as previously described (Mangelsdorf et al., supra). Thus, Northern blot analysis of PPAR mRNA was carried out employing adult and embryonic tissue. Adult male rat tissues and mouse embryos from gestation day 10.5 to 18.5 were employed. The exposure time for each of the blots was 48 hours. The sizes of the transcripts, based on RNA size markers, were 8.5kb (PPAR α), 1.9kb (PPAR γ), and 3.5kb (PPAR δ).

EXAMPLE 4

DNA Binding Assays

Gel mobility shift assays were performed as previously described by Kliewer et al. (1992) supra. PPAR α , PPAR γ , PPAR δ , RXR α , RXR β and RXR γ were synthesized in vitro using the TNT coupled transcription/translation

system (Promega) according to the manufacturer's instructions.

EXAMPLE 5

Isolation of three murine PPAR isoforms

5 The function of peroxisome proliferators has been most extensively studied in rodents, where treatment with these compounds results in marked increases in peroxisome size and number and concomitant increases in the expression of the genes encoding the enzymes of the peroxisomal
10 β -oxidation pathway. To gain insight into the function of PPAR isoforms, mouse embryonic and adult liver libraries were screened for PPAR α -related gene products. In addition to PPAR α , two types of PPAR α -related clones were isolated.

 The first clone encodes a 475-amino acid protein
15 that is 56% identical to mouse (m)PPAR α and 76% identical to *Xenopus* (x)PPAR γ . Since this clone is 97% and 84% identical to the DNA binding and ligand binding domains of xPPAR γ , respectively, it is designated mPPAR γ (see SEQ ID NOs:1 and 2).

20 The second clone encodes a 440-amino acid protein that is closely related to NUC-1 (see SEQ ID NOs:3 and 4, and Figure 1), a PPAR α -related receptor recently isolated from a human osteosarcoma library (see Schmidt et al., in *Mol. Endo.* 6:1634-1641 (1992)). Since this second clone is
25 not highly homologous to any of the previously identified PPAR isoforms (i.e., mPPAR α , xPPAR α , xPPAR β or xPPAR γ ; see Figure 1), it appears to represent a novel receptor, and is, therefore, designated mPPAR δ . Of the approximately 50 positives characterized during the course of screening, no
30 mouse homolog of xPPAR β was identified.

EXAMPLE 6PPAR α , PPAR γ , and PPAR δ are differentially expressed
in the adult and embryo

The expression patterns of the murine PPAR
5 isoforms were examined in the embryo and adult. Northern
analysis of poly(A)⁺ RNA isolated from adult male rat
tissues revealed differential yet overlapping patterns of
expression of the three isoforms. Both PPAR α and PPAR δ are
widely expressed, with PPAR α message levels highest in the
10 liver, kidney, heart, and adrenal, and PPAR δ message
highest in the heart, adrenal, and intestine. In contrast,
PPAR γ displays a more restricted distribution pattern, with
abundant expression in only the adrenal and spleen,
although message is also detectable in the heart, kidney,
15 and intestine.

The developmental expression of the PPAR isoforms
was also examined through Northern analysis of whole mouse
embryo RNA. PPAR α and PPAR γ displayed similar expression
patterns during mouse embryogenesis, with message first
20 appearing at day 13.5 postconception and increasing until
birth. In contrast, PPAR δ message was abundant at all the
embryonic time points tested, suggesting a broad role for
this isoform during development. Thus, the PPAR isoforms
are seen to be differentially expressed in both the embryo
25 and the adult.

EXAMPLE 7Evidence for pharmacological differences between
PPAR α , PPAR γ and PPAR δ

The relatively high degree of conservation within
30 the ligand binding domains of PPAR α , PPAR γ and PPAR δ
suggested that these PPAR isoforms might respond to the
same activators. Accordingly, each of the PPAR isoforms

was first tested for responsiveness to Wy 14,643, a peroxisome proliferator and potent activator of PPAR α (Reddy and Lalwani, *Crit. Rev. Toxicol.* 12:1-58 (1983)). Cotransfection of PPAR α expression plasmid resulted in a dramatic (>100-fold) increase in activation of a reporter construct containing three copies of the acyl-CoA oxidase PPRE (AOX-PPRE) upstream of the thymidine kinase promoter driving luciferase expression (PPRE₃-TK-LUC) in response to Wy 14,643 (Figure 2).

In contrast, no activation of reporter expression was seen in the presence of Wy 14,643 upon cotransfection of PPAR γ or PPAR δ expression plasmids (Figure 2). This lack of activation is unlikely to reflect differences in binding site specificity, as each of the PPAR isoforms bound efficiently to the AOX-PPRE as a heterodimer with RXR (as determined by gel mobility shift assays done using *in vitro* synthesized PPAR α , PPAR γ , and PPAR δ , and/or RXR γ , and ³²P-labeled AOX-PPRE oligonucleotide). Additional experiments revealed that overexpression of PPAR γ and PPAR δ interfered with the ability of PPAR α to activate through the AOX-PPRE (Figure 3). Thus, both PPAR γ and PPAR δ are expressed and can function as dominant repressors of PPAR α -mediated responsiveness to Wy 14,643.

Since no activation of PPAR γ and PPAR δ was detected with Wy 14,643, other potential activators were tested, including a broad spectrum of peroxisome proliferators and fatty acids. As shown in Figure 4, significant activation of PPAR γ was obtained upon treatment with LY-171883, a leukotriene antagonist and peroxisome proliferator which lacks the carboxyl group typically found in this class of compounds (Foxworthy and Eacho, *Biochem. Pharmacology* 42:1487-1491 (1991)). Conversely, no activation of PPAR γ was seen in the presence of linoleic acid (Figure 4).

In contrast to the results obtained with PPAR γ , PPAR δ was activated in the presence of linoleic acid, but was not activated upon treatment with LY-171883. Both LY-171883 and linoleic acid are strong activators of PPAR α (Figure 4). Interestingly, each of the three PPAR isoforms was activated with a distinct rank order of efficacy by these compounds:

PPAR α :

Wy 14,643 > LY-171883 > linoleic acid;

10 PPAR γ :

LY 171883 > linoleic acid > Wy 14,643;

PPAR δ :

linoleic acid > LY-171883 and Wy 14,643.

See Figure 4. These data provide evidence that PPAR γ and
15 PPAR δ can function as regulated activators of gene expression and that the three PPAR isoforms are pharmacologically distinct.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it
20 will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SEQUENCE LISTING

SEQ ID NO:1

Mouse PPAR γ

1 ATCGAATCCCGGCCCCAGGCGCTGCCGCTCTGAGTGGCAGCGGCCCGCGCTGGCGGGCC
 61 GGGAGACCCCGGAAGAAGAGACCTGGGGCGCTGCTGGGGTATTGGGTGCGCGCGCAGTGAG
 121 GCGGACCGAGTGTACGACAAGGTGACCGGGCTGAGGGGACGGGCTGAGGAGAAGTCACAC
 181 TCTGACAGGAGCCTGTGAGACCAACAGCCTGACGGGGTCTCGGTTGAGGGGACGCGGGCT
 241 GAGAAGTCACGTTCTGACAGGACTGTGTACAGACAAGATTGAAAGAACGGGTGACCA
 301 CTGATATTCAGGACATTTTAAAAACAAGACTACCCCTTACTGAAATTACCATGGTTGAC
 M V D 3
 361 ACAGAGATGCCATTCTGCCCCCAACTTCGGAATCAGCTCTGTGGACCTCTCCCTGATG
 T E M F F W P T N F G I S S V D L S V M 23
 421 GAAGACCACTCGCATCTCTTGACATCAAGCCCTTTACCAAGTGTATTCTCCAGCAT
 E D H S H S F D I K P F T T V D F S S I 43
 481 TCTGCTCCAGACTGTGAAGACATTCACACAGAGCTGACCAAGTGTGGTGTCTGATTAC
 S A P H Y E D I P F T R A D P M V A D Y 63
 541 AAATATGACCTGAAGCTCCAAGAATACCAAAGTCCGATCAAAGTGAACCTGCATCTCCA
 K Y D D L K L Q E Y Q S A I K V E P A S P 83
 601 CCTATTATTCTGAAAAGACCCAGCTCTACAAACAGGCTCATGAAGAACCTTTCAACTCC
 P Y Y S E K T Q L Y N R P H E E P S N S 103
 661 CTCATGGCCATTGAGTCCGAGTCTGTGGGGATAAAGCATCAGGCTTCCACTATGGAGTT
 L M A I E C R V C G D K A S G F H Y G V 123
 721 CATGCTGTGAAGGATGCAAGGGTTTTTTCGAGAAGCAATCCGATTGAAGCTTATTAT
 H A C E G C K G F F R R T I R L K L I Y 143
 781 GATAGGTGTGATCTTAACTGCCGATCCACAAAAAAGTAGAAATAAATGTCACTAGT
 D R C D L N C R I H K K S R N K C Q Y C 163
 841 CGGTTTCAGAAGTGCCTTGTCTGTGGGATGTCTCAAAATGCCATCAGGTTGTGGCGGATG
 R F Q K C L A V G M S H N A I R F G R M 183
 901 CCACAGCGCGAAGGAGAAGCTGTTGGCGAGATCTCCAGTGATATCGACAGCTGAAC
 P Q A E K E K L L A E I S S D I D Q L N 203
 961 CCAGAGTCTGCTGATCTGCGAGCCCTGGCAAAGCATTGTATGACTCATATAAAGTCC
 P E S A D L R A L A K H L Y D S Y I K S 223
 1021 TTCGCGCTGACCAAGCCAGGCGGAGCGCATCTTGACAGGAAGACACCGGACAAATCA
 F P A T K K A R A I L T G K T T D K S 243
 1081 CCATTGTGCTATCAGACATGAATTCCTTAATGATGGGAGAAGATAAATCAAGTCAA
 P F V I Y D M N S L M H M G E D K I K F K 263
 1141 CATATCACCCCTGCGAGGACGAGCAAAAGAGTGGCCATCCGAATTTTCAAGGGTGC
 H I T P L Q E Q S K E V A I R I F Q G C 283
 1201 CAGTTTCGATCCGTAGAAGCCGTGCAAGAGATCACAGATATGCCAAAATATCCCTGGT
 Q F R S V E A V Q E I T E Y A K N I P G 303
 1261 TTCAATTAACCTTGATTGAATGACCAAGTACTCTGCTCAAGTATGGTGTCCATGAGATC
 F I N L D L N D Q V T L L K Y G V H E I 323
 1321 ATCTACAGATGCTGGCCCTCCCTGATGAATAAAGATGGAGTCCCTCATCTCAGAGGGCCAA
 I Y T M L A S L M N K D G V L I S E G Q 343
 1381 GGATTCACTGACCGGGAGTTCTCAAAGCCTCGGGAAGCCCTTTGGTGACTTTATGGAG
 G F M T R E F L K S L R K P F G D F M E 363
 1441 CCTAAGTTGAGTTGCTCTGAAGTCAATGCATCGAATTAGATGACAGTGCATGGCT
 P K F E F A V K F N A L E L D D D S D L A 383
 1501 ATATTTATAGCTGTCAATTATCTCAGTGGAGACCGCCAGGCTTGCTGAAGCTGAAGCCC
 I F I A V I I L S G D R P G L L N V K P 403
 1561 ATCGAGGACATCCAAGACAACCTGCTGACGGCCCTGGAAGTGCAGCTCAAGCTGAATCAC
 I E D I Q D N L L Q A L E L Q L K L N H 423
 1621 CCAGAGTCTCTCAGCTGTTGCGCAAGGTGCTCCAGAAGATGACAGACCTCAGGCAGATC
 P E S S Q L F A K K V L Q K M T D L R Q I 443
 1681 GTACAGAGACGCTGACGCTACTGCATGTGATCAAGAAGACAGAGACAGACATGAGCCTT
 V T E H V Q L L H V I K K T E T D M S L 463
 1741 CACCCCTGCTCCAGGAGATCTCAAGGACTTGATTAGCAGGAAGTCCCAACCCGCTGA
 H P L Q E I Y K D L Y * 475
 1801 CACGCTGTCTCTTATGTAATGCACTATTATTGTAGGGAAAAAATCTGACACCTTAAG
 1861 AAATTTACTGTGAAAAGCATTTAAAAACAAAAGTTTTAGAACATGATCTATTTATGC
 1921 ATATTGTTTATAAAGATACATTTACATTTACTTTAATATTTAAAAATTACCACTTATA
 1981 AAAAAAAAAAAAAAAAAAGGAATTC 2005

SEQ ID NO:2

10 25
 M V D T E M P F W P T N F G I S S V D L S V M E D H S H S F D I K P F T T
 50 65
 V D F S S I S A P H Y E D I P F T R A D P M V A D Y K Y D L K L Q E Y Q S
 80 90 105
 A I K V E P A S P P Y Y S E K T Q L Y N R P H E E P S N S L M A I E C R V
 110 130 145
 C G D K A S G F H Y G V H A C E G C K G F F R R T I R N K L I Y D R C D L
 150 160 170 185
 N C R I H K K S R N K C Q Y C R F Q K C L A V G M S H A I R F G R M P Q
 190 200 215
 A E K E K L L A E I S S D I D Q L N P E S A D L R A L A K H L Y D S Y I K
 225 240 255
 S F P L T K A K A R A I L T G K T T D K S P F V I Y D M N S L M M G E D K
 270 285
 I K F K H I T P L Q E Q S K E V A I R I F Q G C Q F R S V E A V Q E I T E
 300 315 325
 Y A K N I P G F I N L D L N D Q V T L L K Y G V H E I I Y T M L A S L M N
 335 345 360
 K D G V L I S E G Q G F M T R E F L K S L R K P F G D F M E P K F E F A V
 375 390 405
 K F N A L E L D D S D L A I F I A V I I L S G D R P G L L N V K P I E D I
 410 425 440
 Q D N L L Q A L E L Q L K L N H P E S S Q L F A K V L Q K M T D L R Q I V
 455 470
 T E H V Q L L H V I K K T E T D M S L H P L L Q E I Y K D L Y

SEQ ID NO:3

Mouse PPAR δ

1 GAATTCCTGGGGATTAATGGGAAAAGTTTGGCaggagCTGGGGGATCTGCGGACCT
 61 GCGGGACggCggCagcGGCGCGAGAGCGCGCGCGgACAGTGTCTGCAGCGGTGTGCGTA
 121 TGCCCATGGGACTCACTCAGAGGCTCTGCTCACTGACAGATGAAGACAAACCCACGGTA
 181 AAGGCAGTCCATCTGCGCTCAGACCCAGATGGTGGCAGAGCTATGACCAGGCTCGACG
 241 CCACGCCAAGTGGGGTTCAGTTCATGGAACGCCACAGGAGGAGACCCCTGAGGCCGGGA
 M E Q P Q E E T P E A R E 13
 301 AGAGGAGAAAGAGGAAGTGGCCATGGGTGACGGAGCCCGAGCTCAATGGGGGACACAGA
 E E K E E V A M G D G A P E L N G G P E 33
 361 ACACAGCCTTCTTCAGCAGCTGTGACAGCTCTCCAGAAATTCCTCCCTCTCCTCCCT
 H T L P S S S C A D L S Q N S P S L 53
 421 CTGGACCGACTGCAGATGGGCTGTGATGGGCTCAGCGGCCAGGCTCAACATGGAATG
 L D Q L Q M G C D G A S G G S L N M E C 73
 481 TCGGCTGTGGGGACAAAGGCTCGGGCTTCCATACGGGTCCACGCGTGGCAGGGGTG
 R V C G D K A S G F H Y G V H A C E G C 93
 541 CAAGGGCTTCTTCGCGCGGACAATCCGATGAAGCTCGATATGAGAAGTGGATCGGAT
 K G F F R R T I R M K L E Y E K C D R I 113
 601 CTGCAAGATCCAGAAGAAGACCGCAACAGTGTGAGTACTGCCGCTTCCAGAGTGCCCT
 C K I Q K K N R N K C Q Y C R F Q K K L 133
 661 GGCACCTCGGCTATGCGACAACGCTATCCGCTTTGGACGGATGCGCGGACGGCGAGAAGAG
 A L G M S H N A I R F G R M P D G E K R 153
 721 GAAGCTGGTGGCGGGCTGACTGCCAGCGAGGGTGCCAGCACAACCCCGAGCTGGCCGA
 K L V A G L T A S E G C Q H N P Q L A D 173
 781 CCTGAAGGCTTCTTAAGCACATCTACAAACGCTACCTGAAAAACTTCAACATGACCA
 L K A F S K H I Y N A Y L K N F N M T K 193
 841 AAGAAGGGCCCGAGCATCTCACCAGCAAGTCCAGCCACAACGACCCCTTTGTATCCA
 K K A R S I L T G K S S H N A P F V I H 213
 901 CGACATCGAGACCTGTGGCAGGACAGAGGGCTGTGTGGAAACAGCTGTGTNAGCG
 D I E T L W Q A E A K G L L V W K Q L V N G 233

32

961 GCTGCCGCCCTACAAACGAGATCAGTGTGCACGTGTTCTACCGCTGCCAGTCCACAGAT
 L P P Y N E I S V H V F Y R C Q S T T V 253
 1021 GGAGACAGTCCGAGAGGTCACCGAGTTCGCCAAGAACATCCCAACTTCAGCAGCTCTT
 E T V R E L T E F A K N I P N F S S L F 273
 1081 CCTCAATGACCAAGGTGACCTCTCTCAAGTATGGCGTGCACGAGGCCATCTTTGCCATGCT
 L N D Q V T L L K Y G V H E A I F A M L 293
 1141 GGCCCTCATCGTCAACAAGACGGGCTGCTGGTGGCCAAACGGCAGTGGCTTCGTCAACCCA
 A S I V N K D G L L V A N G S G F V T H 313
 1201 CGAGTTCTTGGCAAGTCTCCGCAAGCCCTTCAGTGACATCATTGAGCCCAAGTTCGAGTT
 E F L R S L R K P F S D I I E P K F E F 333
 1261 TGCTGTCAAGTTCATGCGCTGGAGCTCGATGACAGTGACCTGGCGCTCTTCATCGCGGC
 A V K F N A L E L D D S D L A L F I A A 353
 1321 CATCATCTGTGTGGAGACGGCCAGGCCCTCATGAATGTGCCCCAGGTAGAAGCCATCCA
 I I L C G D R P G L M N V P Q V E A I Q 373
 1381 GGACACATTCTCGGGGCTCTAGAATTCATCTGCAGGTCAACACCTGACAGCCAGTA
 D T I L R A L E F H L Q V N H P D S Q Y 393
 1441 CCTCTCCCCAAGCTGTGTCAGAAGATGGCAGACCTCGGGCAGCTGTCTACTGAGCATGC
 L F P K L L Q K M A D L R Q L V T E H A 413
 1501 CCAGATGATGACGTGGCTAAAGAAGACGGAGAGTGAGACCTTGCTGCACCCCTGCTCCA
 Q M H Q W L K K T E S E T L L H P L L Q 433
 1561 GGAATCTACAGGACATGTACTAAGCCGagCCcaggcCtCCcCtCagggtCtCgtggy
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 1621 gCCcagCCaCgaCtGTTcAGAGGACcAGCCaAGGcACTGGcAGTCAAGcAGCTAGAGC
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 1741 AACCCCCCAATCCCCCAACCCCCCTCCCCCAAGCTCTCCCCATGGCCCGTTTCCCTG
 1801 TTTCTCCTCAGCACTCTCTGTTCTGTGTCTCCCTAGCGCCTTGCTCCCCCCCCCTTG
 1861 CTTCTCCTCTAGCATCCCCCTCTCCCACTCCTACATTGTGCTGATTACAGCAGAC
 1921 AGCCCGTTGTAGCTCACCAGCAGCCTAAAGCAGTGGGCCGTGTGTGCCCCAGTCTGT
 1981 CCTCTCCTCTATCCCCCTTCAAAGGGAATTC 2012

SEQ ID NO:4

M E Q P Q E E T P E A R E E E K E E V A M G D G A P E L N G G P E H T L P
 S S S C A D L S Q N S S P S S L L D Q L Q M G C D G A S G S L N M E C R
 V C G D K A S G F H Y G V H A C E G C K G F F R R T I R M K L E Y E K C D
 R I C K I Q K K N R N K C Q Y C R F Q K C L A L G M S H N A I R F G R M P
 D G E K R K L V A G L T A S E G C Q H N P Q L A D L K A F S K H I Y N A Y
 L K N F N M T K K K A R S I L T G K S S H N A P F V I H D I E T L W Q A E
 K G L V W K Q L V N G L P P Y N E I S V H V F Y R C Q S T T V E T V R E L
 T E F A K N I P N F S S L F L N D Q V T L L K Y G V H E A I F A M L A S I
 V N K D G L L V A N G S G F V T H E F L R S L R K P F S D I I E P K F E F
 A V K F N A L E L D D S D L A L F I A A I I L C G D R P G L M N V P Q V E
 A I Q D T I L R A L E F H L Q V N H P D S Q Y L F P K L L Q K M A D L R Q
 L V T E H A Q M M Q W L K K T E S E T L L H P L L Q E I Y K D M Y

SEQ ID NO:5

GGNTTYCAYT AYGGNGTNC A YCG 23

SEQ ID NO:6

GFHYGVHA

8

That which is claimed is:

1. An isolated mammalian peroxisome proliferator-activated receptor subunit protein of the γ or δ subtype, or functional fragments thereof.

2. A protein according to claim 1 wherein said protein is of the γ subtype, or functional fragments thereof.

3. A protein according to claim 2 having an amino acid sequence substantially the same as set forth in SEQ ID No:2.

4. A protein according to claim 1 wherein said protein is of the δ subtype, or functional fragments thereof.

5. A protein according to claim 4 having an amino acid sequence substantially the same as set forth in SEQ ID NO:4.

6. A receptor recombinantly expressed in a cell containing a vector comprising isolated nucleic acid encoding a protein according to claim 1.

7. A receptor according to claim 6 comprising at least one PPAR γ subunit or at least one PPAR δ subunit and at least one retinoid X receptor isoform.

8. A receptor according to claim 6, wherein said receptor is a heteromultimer.

9. A receptor according to claim 7, wherein the PPAR subunit is PPAR γ .

10. A receptor according to claim 7, wherein the PPAR subunit is PPAR δ .

11. A mammalian peroxisome proliferator-activated receptor, expressed recombinantly in a host cell, said receptor having the ability to repress PPAR α -mediated responses activated by Wy 14,643, comprising at least one
5 PPAR subunit, wherein the PPAR subunit is PPAR γ or PPAR δ , and at least one retinoid X receptor isoform.

12. A mammalian peroxisome proliferator-activated subunit protein, expressed recombinantly in a host cell comprised of an amino acid sequence shown in SEQ ID NOs: 2 or 4.

13. A method for screening compounds to determine those which bind to mammalian peroxisome proliferator-activated receptor proteins comprising at least one PPAR subunit of the γ or δ subtype, or functional
5 fragments thereof, said method comprising:

contacting receptor protein according to claim 11 with a test compound, and

identifying those compounds which bind to invention receptor proteins.

14. A bioassay for evaluating whether test compounds are agonists for receptors according to claim 11, or functional modified forms of said receptor(s), said bioassay comprising:

- 5 (1) contacting suitable host cells expressing said receptor with test compound under physiological conditions, wherein said host cells contain DNA encoding a reporter protein, wherein said DNA is operatively
10 linked to a PPAR-response element;
- (2) monitoring said host cells for expression of reporter protein, wherein expression of

reporter protein reflects transcriptional activity of said receptor protein and the presence of an activated receptor-ligand complex.

15. A bioassay for evaluating whether test compounds are antagonists for receptors according to claim 11, or functional modified forms of said receptor(s), said bioassay comprising:

- 5 contacting suitable host cells with
- (i) increasing concentrations of at least one compound whose ability to inhibit the transcription activation activity of agonists of mammalian peroxisome proliferator-activated receptors of the γ or δ subtype is sought to be determined, and
- 10 (ii) a fixed concentration of at least one agonist for said receptor protein(s) or functional modified forms thereof,
- 15 wherein suitable host cells express mammalian peroxisome proliferator-activated receptors comprising at least one PPAR subunit of the γ or δ subtype, and wherein said host cells contain DNA encoding a
- 20 reporter protein, wherein said DNA is operatively linked to a PPAR-response element; and thereafter
- assaying for evidence of transcription of said reporter gene in said cells as a function of
- 25 the concentration of said compound in said culture medium, thereby indicating the ability of said compound to inhibit activation of transcription by agonists of mammalian peroxisome proliferator-activated receptors of the γ or δ
- 30 subtype.

16. An antibody generated against the receptor of claim 11.

17. An antibody according to claim 16, wherein said antibody is a monoclonal antibody.

18. A heterodimer complex comprising PPAR γ or PPAR δ and an isoform of RXR.

19. A heterodimer complex according to claim 18 wherein said isoform of RXR is selected from RXR α , RXR β or RXR γ .

20. A combination of receptors comprising at least two different members of the steroid/thyroid superfamily of receptors, wherein said receptors are associated in the form of a multimer, and wherein one of said members is selected from PPAR γ or PPAR δ .

21. A combination of receptors according to claim 20 wherein said combination is in the form of a heterodimer.

22. A multimer comprising at least the dimerization domain of at least two different members of the steroid/thyroid superfamily of receptors, wherein one of said members is selected from PPAR γ or PPAR δ .

23. An isolated nucleic acid encoding a mammalian peroxisome proliferator-activated receptor subunit protein of the γ or δ subtype, or functional fragments thereof.

24. An isolated nucleic acid according to claim 23, wherein said protein is of the γ subtype, or functional fragments thereof.

25. An isolated nucleic acid according to claim 23, wherein said protein is of the δ subtype, or functional fragments thereof.

26. An isolated nucleic acid according to claim 23 having a contiguous nucleotide sequence substantially the same as set forth in SEQ ID NO:1 [PPAR- γ], or SEQ ID NO:3 [PPAR- δ], or variations thereof which encode the same amino acid sequence, but employ different codons for some of the amino acids, or splice variant nucleotide sequences thereof.

27. An isolated nucleic acid according to claim 23 having a contiguous nucleotide sequence substantially the same as set forth in SEQ ID NOs:1 or 3.

28. Isolated and purified nucleic acid, or functional fragments thereof encoding the protein of claim 1, selected from:

(a) nucleic acid encoding the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4, or

(b) nucleic acid that hybridizes to the nucleic acid of (a) under moderately stringent conditions, wherein said nucleic acid encodes biologically active PPAR- γ or PPAR- δ , or

(c) nucleic acid degenerate with respect to either (a) or (b) above, wherein said nucleic acid encodes biologically active PPAR- γ or PPAR- δ .

29. A vector comprising a nucleic acid according to claim 23.

30. A cell containing a nucleic acid according to claim 23.

31. A cell according to claim 30, wherein said cell further comprises nucleic acid encoding at least one retinoid X receptor isoform.

32. A cell containing a vector according to claim 29.

33. A cell according to claim 32, wherein said cell further comprises nucleic acid encoding at least one retinoid X receptor isoform.

34. A method for the recombinant production of a mammalian peroxisome proliferator-activated receptor comprising at least one PPAR subunit of the γ or δ subtype, or functional fragments thereof, said method comprising
5 expressing the nucleic acid of claim 23 in a suitable host cell.

35. A method according to claim 34, wherein the suitable host cell contains nucleic acid encoding at least one retinoid X receptor.

36. A host cell according to claim 35, wherein said host cell is a CV-1 cell.

37. An isolated nucleic acid fragment useful as a hybridization probe, wherein said fragment comprises at least 14 contiguous nucleotides of the nucleic acid according to claim 23, and wherein said fragment is labeled with a detectable substituent.

38. A method to identify clones encoding mammalian peroxisome proliferator-activated receptor subunit proteins of the γ or δ subtype, or functional fragments thereof, said method comprising:

screening a genomic or cDNA library with a nucleic acid probe according to claim 37 under low stringency hybridization conditions, and

5 identifying those clones which display a substantial degree of hybridization to said fragment.

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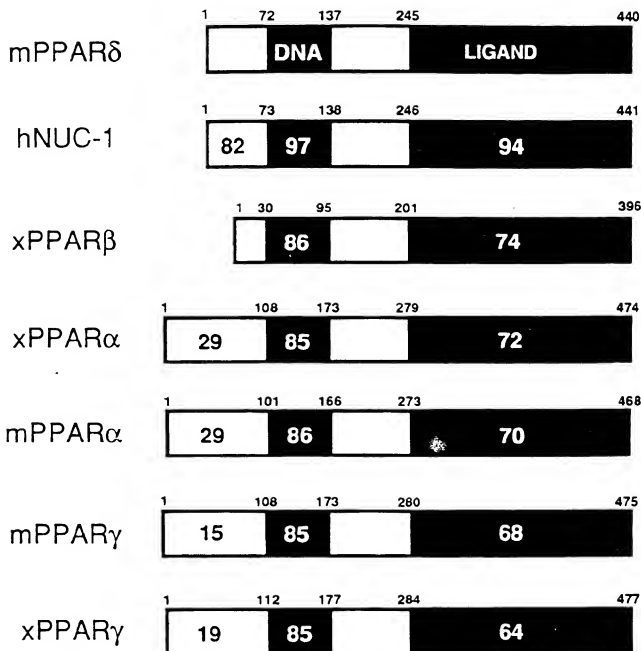


FIG. 1

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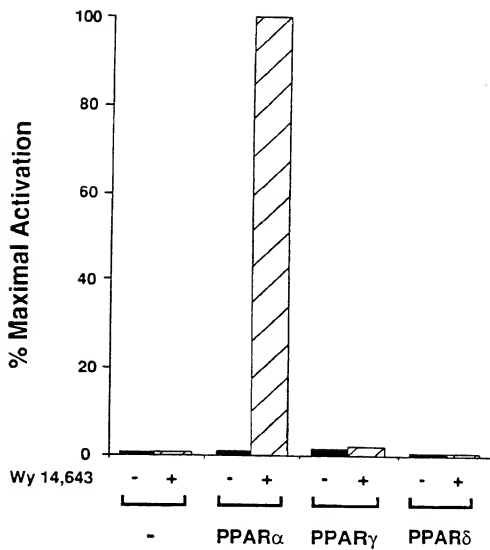


FIG. 2

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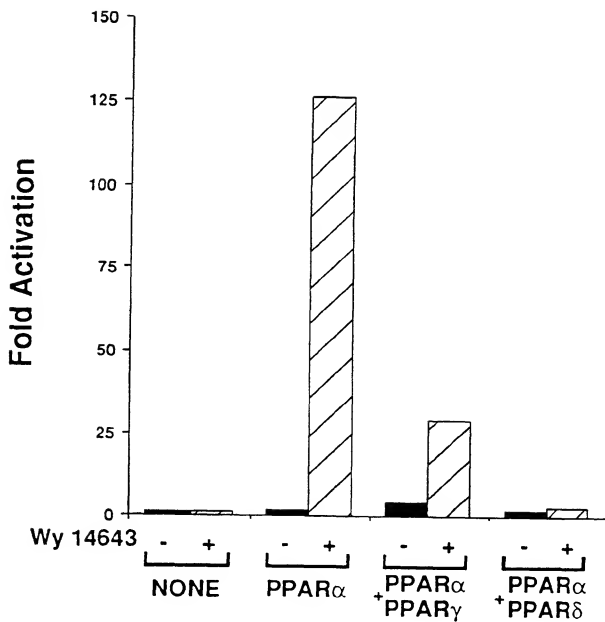


FIG. 3

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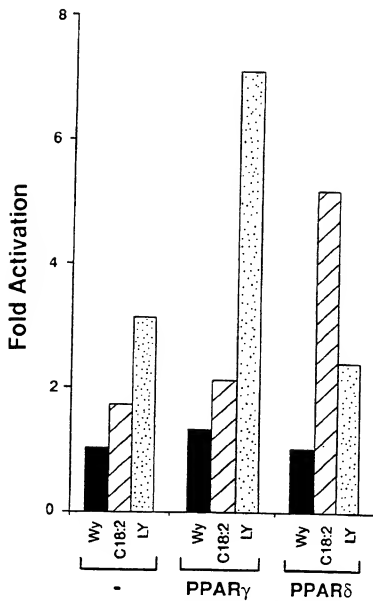


FIG. 4A

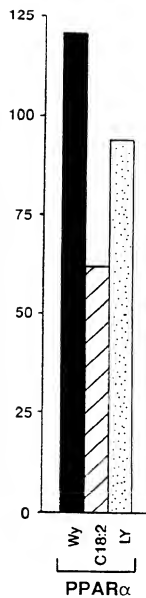


FIG. 4B



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(22) International Filing Date: 27 June 1995 (27.06.95)		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
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(71) Applicant: THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 North Torrey Pines Road, La Jolla, CA 92037 (US).			
(72) Inventors: EVANS, Ronald, M.; 8615 La Jolla Village Drive North, La Jolla, CA 92037 (US). FOREMAN, Barry, M.; Apartment #299, 8568 Via La Jolla, La Jolla, CA 92037 (US). KLIEWER, Steven, A.; 104 Southwick Court, Cory, NC 27513 (US). ONG, Estelita, S.; 6307 Hannon Court, San Diego, CA 92117 (US).			
(74) Agent: REITER, Stephen, E.; Pretty, Schroeder, Brueggemann & Clark, Suite 2000, 444 South Flower Street, Los Angeles, CA 90071 (US).			

(54) Title: MAMMALIAN PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS AND USES THEREOF

(57) Abstract

Peroxisome proliferator-activated receptor subunits designated PPAR γ and PPAR δ are described. Nucleic acid sequences encoding the receptor subunits, expression vectors containing such sequences and host cells transformed with such vectors are also disclosed, as are heterodimeric PPAR receptors comprising at least one of the invention subunits, and methods for the expression of such novel receptors, and various uses therefor.

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INTERNATIONAL SEARCH REPORT

Inter. Application No.

PCT 95/08193

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/85 C07K14/705 C07K16/28 C12N5/10
 C12Q1/80 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 36, 25 December 1993 MD US, pages 26817-26820, YIJUN ZHU ET AL. 'Cloning of a new member of the peroxisome proliferator-activated receptor gene family from mouse liver' see abstract see page 26818, left column, paragraph 5 - right column, paragraph 2 see page 26818, right column, paragraph 4 - page 26820, right column, paragraph 1; figure 1 --- -/-	1-3,6, 12-14, 23,24, 26-30, 32,34-36

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 196, no. 2, 29 October 1993 ORLANDO, FL US, pages 671-677, FANG CHEN ET AL. 'Identification of two mPPAR related receptors and evidence for the existence of five subfamily members' see abstract see page 673, paragraph 2 - page 674, paragraph 3; figures 1,2 see page 676, paragraph 2</p> <p>---</p>	1-5, 23-28, 37,38
X	<p>WO,A,93 06215 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 1 April 1993</p> <p>see page 1, line 33 - page 2, line 8 see page 4, line 19 - page 5, line 28 see page 9, line 9 - line 28 see page 11, line 34 - page 12, line 9 see page 13, line 22 - page 19, line 27 see page 23, line 30 - page 24, line 25 see SEQ ID Nos. 9, 10</p> <p>---</p>	1,4-8, 10, 12-14, 18-23, 25-35, 37,38
X	<p>GB,A,2 265 276 (MERCK & CO INC) 29 September 1993</p> <p>see page 3, line 21 - page 19, line 10</p> <p>---</p>	1,4-8, 10, 12-14, 18-23, 25-35, 37,38
P,X	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, no. 15, 19 July 1994 WASHINGTON US, pages 7355-7359, S.A. KIEWER ET AL. 'Differential expression and activation of a family of murine peroxisome proliferator-activated receptors' see abstract see page 7355, right column, paragraph 2 - paragraph 3 see page 7356, right column, paragraph 2; figure 1 see page 7356, right column, last paragraph - page 7358, left column, paragraph 1</p> <p>-----</p>	1-6, 11-14, 23-30, 32,34-36

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Inter national Application No.

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